Amendments to the Specification

Please note that insertions are indicated by double underlining in order to differentiate between insertions made by way of the current amendment, and underlining present in the specification as originally filed.

A. Please replace the paragraph at page 35 line 19-page 36 line 2 with the following amended paragraph:

For instance, the peptide addition for use in the present invention can comprise a peptide sequence selected from the group consisting of INA[T/S], GNI[T/S], VNI[T/S], SNI[T/S], ASNI[T/S] (SEQ ID NO:8), NI[T/S], SPINA[T/S] (SEQ ID NO:9), ASPINA[T/S] (SEQ ID NO:10), ANI[T/S]ANI[T/S]ANI (SEQ ID NO:11), ANI[T/S]GSNI[T/S]GSNI[T/S] (SEQ ID NO:12), FNI[T/S]VNI[T/S]V (SEQ ID NO:114), YNI[T/S]VNI[T/S]V (SEQ ID NO:115), AFNI[T/S]VNI[T/S]V (SEQ ID NO:116), AYNI[T/S]VNI[T/S]V (SEQ ID NO:117), APND[T/S]VNI[T/S]V (SEQ ID NO:118), ANI[T/S], ASNS[T/S]NNG[T/S]LNA[T/S] (SEQ ID NO:13), ANH[T/S]NE[T/S]NA[T/S] (SEQ ID NO:14), GSPINA[T/S] (SEQ ID NO:15), ASPINA[T/S]SPINA[T/S] (SEQ ID NO:16), ANN[T/S]NY[T/S]NW[T/S] (SEQ ID NO:17), ATNI[T/S]LNY[T/S]AN[T/S]T(SEQ ID NO:18), AANS[T/S]GNI[T/S]NG[T/S] (SEQ ID NO:19), AVNW[T/S]SND[T/S]SNS[T/S] (SEQ ID NO:20), GNA[T/S], AVNW[T/S]SND[T/S]SNS[T/S] (SEQ ID NO:21), ANN[T/S]NY[T/S]NS[T/S] (SEQ ID NO:22), ANNTNYTNWT (SEQ ID NO:23), ANI[T/S]VNI[T/S]V (SEQ ID NO:119), ND[T/S]VNF[T/S] (SEQ ID NO:120) and NI[T/S]VNI[T/S]V (SEQ ID NO:121) wherein [T/S] is either a T or an S residue, preferably a T residue. Other non-limiting examples include a peptide addition comprising the sequence NSTQNATA (SEQ ID NO:112), which corresponds to positions 231 to 238 of the human calcium activated channel 2 precursor (to add two N-glycosylation sites), or the sequence ANLTVRNLTRNVTV (SEQ ID NO:113), which corresponds to positions 538 to 551 of the human G protein coupled receptor 64 (to add three N-glycosylation sites).

B. Please replace the paragraph at page 36 lines 8-11 with the following amended paragraph:

In a more specific embodiment, the peptide addition X is selected from the group consisting of INA[T/S], GNI[T/S], VNI[T/S], SNI[T/S], ASNI[T/S] (SEQ ID NO:8), NI[T/S], SPINA[T/S] (SEQ ID NO:9), ASPINA[T/S] (SEQ ID NO:10), ANI[T/S]ANI[T/S]ANI (SEQ ID NO:11), and ANI[T/S]GSNI[T/S]GSNI[T/S] (SEQ ID NO:12), wherein [T/S] is either a T or an S residue, preferably a T residue.

C. Please replace the paragraphs at page 61 lines 2-16 with the following amended paragraphs:

Sequence of primers used for cloning the wt GCB coding region and inserting signal peptides into the pGCBmat plasmid as described in Example 1.

SO49 (WT-sp-BglII; SEQ ID NO:25): 5'-
CGCAGATCTGATGGCTGGCAGCCTCACAGGATTGC-3'

SO50 (WT-stop-EcoRI; SEQ ID NO:26): 5'-
CCGGAATTCCCATCACTGGCGACGCCACAGGTAGGTG-3'

SO51 (WT-mature-SacI; SEQ ID NO:27): 5'-
ACGCGAGCTCGCCCCTGCATCCCTAAAAGCTTCGG-3'

SO52 (SPegt-NheI/SacI-as; SEQ ID NO:28): 5'-
GCGTTGACGGCAGTCAGAGTTGACAGAAGGGCCAGCCAGCAAAGGATAGTCATG-3'

SO53 (SPegt-NheI/SacI-s; SEQ ID NO:29): 5'-
CTAGCATGACTATCCTTTGCTGGCTGGCCCTTCTGTCAACTCTGACTGCCGTCAACGC
AGCT-3'

SO54 (SPegt-NheI/SacI-as; SEQ ID NO:30): 5'-
CCTGCTACTGCTCCCAGCAGCAGTGAAAGAGTCCAAAGTGGCAGCATG-3'

SO55 (SPegt-NheI/SacI-s; SEQ ID NO:31): 5'-
CTAGCATGCTGCCACTTTGGACTCTTTCACTGCTGCTGGGAGCAGTAGCAGGAGCT-3'

D. Please replace the paragraphs at page 64 line 6 - page 65 line 15 with the following amended paragraphs:

Nucleotide sequences encoding the following N-terminal peptide additions were added to the nucleotide sequence shown in SEQ ID NO 2 encoding wtGCB: (A-4)+(N-3)+(I-2)+(T-1) (representing an extension to the N-terminal of the amino acid sequence shown in SEQ ID NO 1 with the amino acid residues ANIT; SEQ ID NO:33), and (A-7)+(S-6)+(P-5)+(I-4)+(N-3)+(A-2)+(T-1) (ASPINAT; SEQ ID NO:34).

A nucleotide sequence encoding the N-terminal peptide addition (A-4)+(N-3)+(I-2)+(T-1) was prepared by PCR using the following conditions:

PCR 1:

Template: 10 ng pBlueBac5 with wt GCB cDNA sequence

primer SO60 (SEQ ID NO:32): 5'-CAGCTGGCCATGGGTACCCGG-3' and

primer SO85 (SEQ ID NO:35):

5'-TGGGCATCAGGTGCCAACATTACAGCCCGCCCCTGCATCCCTAAAAGC-3'

BIO-X-ACT™ DNA polymerase (Bioline, London, U.K.)

1xOptiBuffer™ (Bioline, London, U.K.)

30 cycles of 96°C 30s, 55°C 30s, 72°C 1 min

PCR 2:

Template: 10 ng pBlueBac5 with wt GCB,

Baculo virus forward primer (SEQ ID NO:36): 5'-TTTACTGTTTTCGTAACAGTTTTG-3' and

PrimerSO86 (SEQ ID NO:37):

5'- GCAGGGGCGGGCTGTAATGTTGGCACCTGATGCCCACGACACTGCCTG-3'

BIO-X-ACT™ DNA polymerase (Bioline, London, U.K.)

1xOptiBuffer™ (Bioline, London, U.K.)

30 cycles of 96°C 30s, 55°C 30s, 72°C 1 min

PCR 3:

3 µl of agarose gel purified PCR1 and PCR2 products (app. 10 ng)

Baculo virus forward primer (SEQ ID NO:36): 5'-TTTACTGTTTTCGTAACAGTTTTG-3' and primer SO60.

BIO-X-ACT™ DNA polymerase (Bioline, London, U.K.)

1xOptiBuffer™ (Bioline, London, U.K.)

30 cycles of 96°C 30s, 55°C 30s, 72°C 1 min

PCR 3 was agarose gel purified and digested with NheI and NcoI and cloned into pBluebac4.5+wtGCB digested with NheI and NcoI.

After confirmation of the correct mutations by DNA sequencing the plasmid was transfected into insect cells using the Bac-N-Blue™ transfection kit from Invitrogen, Carlsbad, CA, USA. Expression of the muteins was tested by western blotting and by activity measurement of the muteins using the GCB Activity Assay.

Enzymatic activity of wtGCB (SEQ ID NO 1) expressed in the expression vector pVL1392 in insect cells (Sf9) using an analogous method to that described in Example 1 gave 13 units/L, while the N-terminal peptide addition ASPINAT (SEQ ID NO:34) gave 28.5 units/L.

E. Please replace the paragraphs at page 65 line 18 - page 66 line 6 with the following amended paragraphs:

Using random mutagenesis two different libraries were constructed on the basis of GCB polypeptides with an N-terminal extension - library A with an N-terminal extension encoding the following amino acid sequence AXNXTXNXTXNXT (SEQ ID NO:38), and library B with an N-terminal extension encoding ANXTNXTNXT (SEQ ID NO:39).

Primers for library A were designed:

SO167 (SEQ ID NO:40): 5'-GTGTCGTGGGCATCAGGTGCCNN(G/C)AA(C/T)(T/A/G)N(G/C)AC(A/T/C)(T/A/G)N(G/C)AA(C/T)(T/A/G)N(G/C)AC(A/T/C)(T/A/G)N(G/C)AA(C/T)(T/A/G)N(G/C)AC(A/T/C)GCCCGCCCCTGCATCCCTAAAAGC

SO168 (SEQ ID NO:41): 5'-GGCACCTGATGCCCACGACACTGCCTG

Primers for library B were designed using trinucleotides in the random positions. X is a mixture of trinucleotide codons for all natural amino acid residues, except proline. The trinucleotide codons used were the same as described by Kayushin et al., Nucleic Acids Research, 24, 3748-3755, 1996.

SO165 (SEQ ID NO:42): 5'-CGTGGGCATCAGGTGCCAAC(X)AC(A/T/C)AA(C/T)(X)AC(A/T/C)AA(C/T)(X)AC(A/T/C)GCCCGCCCCTGCATCCCTAAAAGC

SO166 (SEQ ID NO:43): 5'- GTTGGCACCTGATGCCCACGACACTGCCTG

For both libraries:

SO60 and pBR10 (SEQ ID NO:36): 5'- TTT ACT GTT TTC GTA ACA GTT TTG

F. Please replace the paragraphs at page 67 line 4 - page 70 line 15 with the following amended paragraphs:

PCR 3A and 3B were agarose gel purified and digested with NheI and NcoI and ligated into pGC-12 digested with NheI and NcoI. The ligation mixture is transformed into competent E. coli. The diversity of the library was examined by DNA sequencing of different E. coli clones and gave rise to the following amino acid sequences:

Library A:

1: AFNXTLNKTWN(F/L)T (SEQ ID NO:44)

2: TMNNTWNWTWNWT (SEQ ID NO:45)

3: -EXT wt

4: ALNSTGNLTVDGT (SEQ ID NO:46)

5: ASNSTFNLTENLT (SEQ ID NO:47)

6: TRNVTINCTUNST (SEQ ID NO:48)

7: -EXT wt

8: ALNWTYNGTKNVT (SEQ ID NO:49)

9: AANWTVNFTGNFT (SEQ ID NO:50)

10: -EXT wt

11: AXNXTVNSTUNVT (SEQ ID NO:51)

12: ANNFTFNGTLNLT (SEQ ID NO:52)

13: AGNWTANVTVNVT (SEQ ID NO:53)

14: AGNSTSNVTGNWT (SEQ ID NO:54)

15: AVNSTMNfHAIPP (1 deletion - nonsens) (SEQ ID NO:55)

16: AGNGTVNGTINGT (SEQ ID NO:56)

17: AVNSTGNXTGNWT (SEQ ID NO:57)

18: AGNGTUNGTSNLT (SEQ ID NO:58)

19: -EXT wt

20: AMNSTKNSTLNIT (SEQ ID NO:59)

21: AFNYTSKNST (SEQ ID NO:60)

22: -EXT wt

23: AVNATMNWTANGT (SEQ ID NO:61)

24: ASNSTNNGTLNAT (SEQ ID NO:62)

25: ARNKTKNFTINLT (SEQ ID NO:63)

26: APNITUNDTVNMT (SEQ ID NO:64)

27: AQNKTFNFTMNCT (SEQ ID NO:65)

28: ALNVTWNCTLNLT (SEQ ID NO:66)

29: ALNTTWTNLT (SEQ ID NO:67)

Library B:

1: ANTTNFTNET (SEQ ID NO:68)

2: ANWTNRTNCT (SEQ ID NO:69)

3: ANWTNFTNWT (SEQ ID NO:70)

4: PTGLIGTNFT (SEQ ID NO:71)

5: ANWTNKTNFT (SEQ ID NO:72)

6: ANNTNLTNAT (SEQ ID NO:73)

7: ANYTNWTNFT (SEQ ID NO:74)

8: ANTTNQTNDT (SEQ ID NO:75)

9: - EXT wt

10: ANRTNWTNTT (SEQ ID NO:76)

11: PTATNHTNST (SEQ ID NO:77)

12: - EXT wt

13: ANWTNQTNQT (SEQ ID NO:78)

14: ANWTNWTNAT (SEQ ID NO:79)

15: ANFTNKTNMT (SEQ ID NO:80)

16: ANHTNETNAT (SEQ ID NO:81)

17: AN(C/W)TNFTNET (SEQ ID NO:82)

18: ANLDKLHKUH (insertion - nonsens) (SEQ ID NO:83)

19: ANCFTNQTNFT (SEQ ID NO:84)

20: ANWTNWTNEWT (SEQ ID NO:85)

21: ANCTNWTNCT (SEQ ID NO:86)

22: - EXT wt

23: - EXT wt

24: CHPYNWTNWT (SEQ ID NO:87)

25: ANETNYTNET (SEQ ID NO:88)

26: ANWTNWT (SEQ ID NO:89)

27: AKPYKSYKFY (insertion - nonsens) (SEQ ID NO:90)

28: ANITNKTNWT (SEQ ID NO:91)

29: ANWTNMTNIT (SEQ ID NO:92)

30: ANNTNRTNFT (SEQ ID NO:93)

31: ANWTNWTNWT (SEQ ID NO:94)

32: ANWRTNHTNKT (SEQ ID NO:95)

33: - EXT wt

34: ANQTNITNWT (SEQ ID NO:96)

Library B was transfected into insect cells using the Bac-N-Blue™ transfection kit from Invitrogen, Carlsbad, CA, USA. First, 96 plaques from Library B were picked and tested by activity measurement (GCB Activity Assay). Plaques were selected as follows: 3 with high activity, 3 with medium activity and 3 with low or no activity, and virus was purified for DNA sequencing resulting in the following amino acid sequences:

High activity:

1-1: Mixed sequence

1-2: ANFTNVATNQT (SEQ ID NO:97)

1-3: (A)(N)TTXLTN(K)T (SEQ ID NO:98)

Medium activity:

2-1: ANKTN(S/C)TNIT (SEQ ID NO:99)

2-2: Mixed sequence

2-3: ANWTNCTN(I)T (SEQ ID NO:100)

Low activity:

3-1: ANWTN(F/L)TNWT (SEQ ID NO:101)

3-2: CQLDURSTNET (SEQ ID NO:102)

3-3: No sequence

From both libraries 96 plaques were picked and tested by activity measurement (GCB Activity Assay). From each library 6 plaques with high activity were selected and virus were purified for DNA sequencing. The amino acid sequence encoded by the different clones were:

Library A:

1: Mixed sequence

2: Mixed sequence

3: Mixed sequence

4: WT

5: ANNTNYTNWT (SEQ ID NO:103)

6: ANNTNYTNWT (SEQ ID NO:104)

Library B:

1: AANDTUNWTVNCT (SEQ ID NO:105)

2: ATNITLNYTANTT (SEQ ID NO:106)

3: WT

4: AANSTGNITINGT (SEQ ID NO:107)

5: AVNWTSNDTSNST (SEQ ID NO:108)

G. Please replace the table between page 71 line 2 and page 71 line 4 with the following amended table:

Plasmid	Vector	Mutations	# Glycosylation sites introduced	Activity after Plaque Isolation (U/L)
pGC-1	PBlueBac4.5	Wt	0	6
pGC-6	pBlueBac4.5	N-term: ANIT (SEQ ID NO:33)	1	3
pGC-12	pVL1392	Wt	0	13
pGC-13	pVL1392	N-term: ASPINAT (SEQ ID NO:34)	1	29
pGC-36	pVL1392	N-term: ASPINATSPINAT (SEQ ID NO:109)	2	16
pGC-38	pVL1392	N-term: ASPINAT,K194N, K321N	3	16
pGC-40	pVL1392	N-term: ASPINAT,T132N, K293N, V295T	3	3.5
pGC-47	pVL1392	N-term: AGNGTVNGTINGT (SEQ ID NO:56)	3	30
pGC-48	pVL1392	N-term: ASNSTNNGTLNAT (SEQ ID NO:13)	3	36
pGC-56	pVL1392	N-term: ASPINATSPINAT, K194N, K321N	4	24
pGC-57	pVL1392	N-term: ASPINAT, T132N, K194N, K321N	4	20
pGC-58	pVL1392	N-term: ASPINAT, T132N, K194N	3	10
pGC-60	pVL1392	N-term: ANNTNYTNWT (SEQ ID NO:23)	3	P2: 14
pGC-61	pVL1392	N-term: ATNITLNYTANTT (SEQ ID NO:18)	3	P2: 38
pGC-62	pVL1392	N-term: AANSTGNITINGT (SEQ ID NO:19)	3	P2: 35

pGC-63	pVL1392	N-term: AVNWTSNDTSNST (SEQ ID NO:21)	3	P2: 66
pGC-68	pVL1392	AN N-term extension + R2T	1	37

H. Please replace the paragraphs at page 72 between lines 7 and 9 with the following amended paragraphs:

The 6 GCB polypeptide variants were:

GC-36: ASPINATSPINAT(SEQ ID NO:109)-GCB,

GC-38: ASPINAT(SEQ ID NO:34)-GCB(K194N,K321N),

GC-60: ANNTNYTNWT(SEQ ID NO:23)-GCB,

GC-61: ATNITLNYTANTT(SEQ ID NO:18)-GCB,

GC-62: AANSTGNITINGT(SEQ ID NO:19)-GCB, and

GC-63: AVNWTSNDTSNST(SEQ ID NO:21)-GCB.

I. Please replace the paragraph at page 81, lines 4-12 with the following amended paragraph:

A construct encoding a modified form of FSH-alpha, having two additional sites for N-linked glycosylation at its N-terminus was generated by site-directed mutagenesis using standard DNA techniques known in the art. A DNA fragment encoding the sequence Ala-Asn-Ile-Thr-Val-Asn-Ile-Thr-Val (SEQ ID NO:119) was inserted immediately upstream of the mature FSH-alpha sequence in pBvdH977. The sequence of the resulting plasmid, termed pBvdH1163, is given in SEQ ID NO:7 (modified FSH-alpha-encoding sequence at position 1225 to 1599). A plasmid encoding both subunits was constructed by subcloning the FSH-containing *Nru*I-*Pvu*II fragment from pBvdH1163 into pBvdH1022 (Example 4), which had been linearized with *Pvu*II. The resulting plasmid was termed pBvdH1208.

J. Please replace the paragraph at page 82, lines 1-7 with the following amended paragraph:

FSH1208 was purified and characterized as described in Example 5. SDS-PAGE, run under non-dissociating conditions (without boiling), showed FSH1208 migrating as an apparent 55±5 kDa band, slightly diffuse due to heterogeneity in the attached carbohydrates. The purity was about 80-90%. N-terminal sequencing showed that while the β-chain had the same N-terminal sequence as wildtype FSH, the sequence of α-chain was in agreement with this subunit carrying the expected N-terminal extension ANITVNITV (SEQ ID NO:119), in which both asparagines residues are glycosylated.

K. Please replace the paragraphs at page 82 line 26 - page 83 line 3 with the following amended paragraphs:

Plasmids encoding variant forms of FSH-alpha and FSH-beta containing additional sites for N-linked glycosylation were generated by site-directed mutagenesis using standard DNA techniques known in the art. The following amino acid substitutions and/or insertions were generated:

FSH1147: Amino acid Tyr58 of mature FSH-beta altered to Asn

FSH1349: N-terminus of mature FSH-alpha altered from APDVQDC (amino acids 1-7 of SEQ ID NO:5)... to: APNDTVNFT QDC (SEQ ID NO:122)...

FSH1354: N-terminus of mature FSH-beta altered from NS CEL (amino acids 1-5 of SEQ ID NO:6)... to: NSNITVNITV CEL (SEQ ID NO:123)...